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Toll like receptor 2 knock-out attenuates carbon tetrachloride (CCl_4)-induced liver fibrosis by downregulating MAPK and NF-κB signaling pathways

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ABSTRACT

Innate immune signaling associated with Toll-like receptors (TLRs) is a key pathway involved in the progression of liver fibrosis. In this study, we reported that TLR2 is required for hepatic fibrogenesis induced by carbon tetrachloride (CCl_4). After CCl_4 treatment, $TLR2^{-/-}$ mice had reduced liver enzyme levels, diminished collagen deposition, decreased inflammatory infiltration and impaired activation of hepatic stellate cells (HSCs) than wild type (WT) mice. Furthermore, after CCl_4 treatment, $TLR2^{-/-}$ mice demonstrated downregulated expression of profibrotic and proinflammatory genes and impaired mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) activation than WT mice. Collectively, our data indicate that TLR2 deficiency protects against CCl_4-induced liver fibrosis.

1. Introduction

Liver fibrosis, as the final common endstage of most chronic liver diseases [1], is triggered by chronic liver injury caused by various etiologies including viral infection, cholestasis, metabolic diseases and alcohol abuse [2,3]. It is a reversible wound-healing response characterized by the accumulation of extracellular matrix proteins (ECM) including collagen [4]. Hepatic stellate cells (HSCs) are the major source of extracellular matrix components and can transdifferentiate into hepatic myofibroblasts closely involved in proliferation and collagen synthesis. Moreover, the "activated" HSCs have been shown to contribute to expression of α-smooth muscle actin (α-SMA) and profibrotic cytokines such as transforming growth factor β1 (TGF-β1) and platelet-derived growth factor (PDGF) [5]. Additionally, the inflammatory cytokines (e.g., IL-6) produced by Kupffer cells, the liver resident macrophage, aggravate liver fibrosis [6]. The correlation between inflammation and fibrosis progression has been increasingly clarified recently [7]. However, little is known about the direct biological effect of inflammatory mediators on liver fibrogenesis. Since the demonstration that even advanced liver fibrosis is reversible [8], there is a dire need to uncover the molecular mechanisms underlying the association between liver inflammation and fibrogenesis.

Toll like receptors (TLRs), a class of pattern recognition receptors that can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), play a specific role in the regulation of inflammation [9,10]. Upon activated by agonists, TLRs initiate a signaling cascade resulting in the stimulation of innate and adaptive immune responses [11]. TLR2 acts as a receptor for cell-wall components of gram-positive

Abbreviations: CCl_4, carbon tetrachloride; α-SMA, smooth muscle alpha-actin; WT, wild-type; TGF, transforming growth factor; Col1A1, type I procollagen α1 chain; TLR, Toll like receptor; TBIL, total bilirubin; ALP, alkaline phosphatase; MAPK, mitogen-activated protein kinases; IL, interleukin; NF-κB, nuclear factor kappa B; TNF, tumor necrosis factor; H&E, hematoxylin and eosin; HSC, hepatic stellate cell; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ECM, extracellular matrix proteins; PDGF, platelet-derived growth factor; PAMP, pathogen-associated molecular patterns; DAMP, damage-associated molecular patterns; LTA, lipoteichoic acid

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bacteria (peptidoglycan, PGN and lipoteichoic acid, LTA), which play a significant role in the pathogenesis of severe inflammatory responses [12,13]. Accumulating evidence has indicated that TLR2 signal can not only activate the ASK1/p38 MAPK/NF-KB signaling pathway, but can also stimulate the ERK/JNK and PI3K/Akt pathways in a MyD88-independent manner [14]. On the other hand, growing number of researches have proved that the gram-positive bacteria products can initiate and perpetuate HSCs activation during liver inflammation and fibrosis by upregulating the TLRs level [15,16]. An important finding from Seki et al. was that TLR4, but not TLR2, are required for hepatic fibrogenesis [7]. However, a contradictory conclusion was drawn by Miura et al. [17] who reported that TLR2 promoted liver inflammation and fibrogenesis in non-alcoholic steatohepatitis (NASH) induced by a methionine- and choline-deficient (MCD) diet. Therefore, the role of TLR2 concerning liver fibrosis progression needs additional evidence.

In this study, we investigated the effect of TLR2 deficiency on hepatic fibrosis induced by carbon tetrachloride (CCl_4). Our findings showed that liver fibrosis was ameliorated in $TLR2^{-/-}$ mice as compared with wild type (WT) littermates in this model.

2. Materials and methods

2.1. Animals and mouse model of liver fibrosis

$TLR2^{-/-}$ mice on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) C57BL/6J mice were from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). Male 8–10 weeks old (25–30 g) WT and $TLR2^{-/-}$ mice were housed in a specific pathogen free facility with a 12-h light/dark cycle, and fed *ad libitum* with regular chow food and water throughout the study period. Chronic CCl_4-dependent liver fibrosis was induced by intra-peritoneal CCl_4 (diluted to 20% with corn oil) administrated as a dose of 6 μl/g body weight 3 times a week for 6 weeks. The control groups received the same volume of vehicle [7]. All animals were sacrificed at one day after the final injection (with fasting), and received care according to the guidelines published by National Institutes of Health and approved by the local ethics committee of Fudan University.

2.2. Biochemical assays

All serum samples from individual mice were obtained and stored at −80 °C until use. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBIL) levels were determined using the commercial kits (Nanjing Jiancheng Biotechnology Institute, China).

2.3. Histological analyses

For morphometric analyses, the liver tissues maintained in 10% buffered formalin and embedded in paraffin were sliced into 4 μm-thick sections. Then, the sections were stained with hematoxylin and eosin (H&E) to monitor histological changes. To evaluate collagen deposition, Masson's trichrome staining and Sirius red staining were performed. For detection of hepatic stellate cell activation and inflammatory infiltration, all slides were stained with monoclonal antibodies smooth muscle alpha-actin (α-SMA, Sigma) and F4/80 (AbD Serotech, Raleigh, NC). Histological analyses were performed according to standard procedures described previously [18]. Besides, the extent of fibrosis was quantified by measuring percentages of Sirius red-positive pixels and counting the number of α-SMA positive HSCs in the sinusoidal lining area in five randomly selected 200- or 400-fold magnification fields per liver section [18].

2.4. Real-time PCR analysis

Total RNA from livers was prepared with Trizol reagent (Invitrogen) in accordance with the manufacturer's protocol. Subsequently, reverse transcription and quantitative real-time PCR were implemented using commercial kits (TaKaRa) according the manufacturer's instruction. And an ABI StepOne plus Sequence Detection system was used to detect a series of changes of mRNA levels associated with liver fibrosis. Meanwhile, all final results were normalized relative to a housekeeping gene, GAPDH. The primers were shown in Table 1.

2.5. Western blotting and nuclear factor kappa B (NF-κB) activity measurements

Western blotting was carried out as described [19]. Primary antibodies used included those against α-SMA (Sigma), GAPDH (Santa Cruz Biotechnology), pThr202/pTyr204-ERK1/2, ERK1/2, pThr183/pTyr185-JNK, JNK, pThr180/pTyr182-p38, p38 (Cell Signaling Technology). Nuclear extracts were prepared according to the Nuclear Extract Kit (Active Motif). NF-κB activation was assessed in nuclear extracts by means of the NF-κB Transcription Factor Assay Kit (TransAM™) from Active Motif as described [20].

2.6. Statistical analysis

All results were expressed as mean ± SEM. Comparisons between different groups were assessed by the Student *t* test and one-way ANOVA analysis using GraphPad Prism 5 (GraphPad Software). $P < 0.05$ was considered significant.

Additional materials and methods are shown in Supplemental information.

3. Results

3.1. TLR2 deficiency reduces liver injury and collagen accumulation upon CCl_4 administration

We determined the serum levels of liver enzymes including ALT, AST, ALP, and TBIL. As shown in Fig. 1A, 6 weeks injection of CCl_4 led to a marked elevation in ALT, AST, ALP, and TBIL levels. TLR2 deficiency had no effect on CCl_4-induced elevation in serum ALT and AST levels. However, TLR2 deficiency suppressed the CCl_4-induced increases in serum ALP and TBIL levels. In addition, histological analysis revealed that hepatocellular necrosis and infiltration of inflammatory cells aroused by prolonged CCl_4

Table 1
Primer sequences used for real-time PCR.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Col1α1	GAGAGGTGAACAAGGTCCCG	AAACCTCTCTCGCCTCTTGC
α-SMA	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA
TNF-α	AGCCGATGGGTTGTACCTTG	ATAGCAAATCGGCTGACGGT
TGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
MCP-1	ATGCAGTTAACGCCCCACTC	CCCATTCCTTCTTGGGGTCA
TIMP1	CCAGAACCGCAGTGAAGAGT	GTACGCCAGGGAACCAAGAA
Ccl4	CCCAGCTCTGTGCAAACCTA	CCATTGGTGCTGAGAACCCT
PDGFR	CGAAAACTGTCACCCACACC	GTGACCTCCTGCGAATCTCC
IL-6	GAGTGGCTAAGGACCAAGACC	AACGCACTAGGTTTGCCGA
TLR4	GGTGTGAAATTGAGACAATTGA	GTTTCCTGTCAGTACCAAGGTTG
CXCL-1	GCTGGGATTCACCTCAAGAA	TGGGGACACCTTTTAGCATC
CXCL-2	CGCTGTCAATGCCTGAAGAC	ACACTCAAGCTCTGGATGTTCTT
GAPDH	TGCCGCCTGGAGAAACCT	TGAAGTCGCAGGAGACAACC

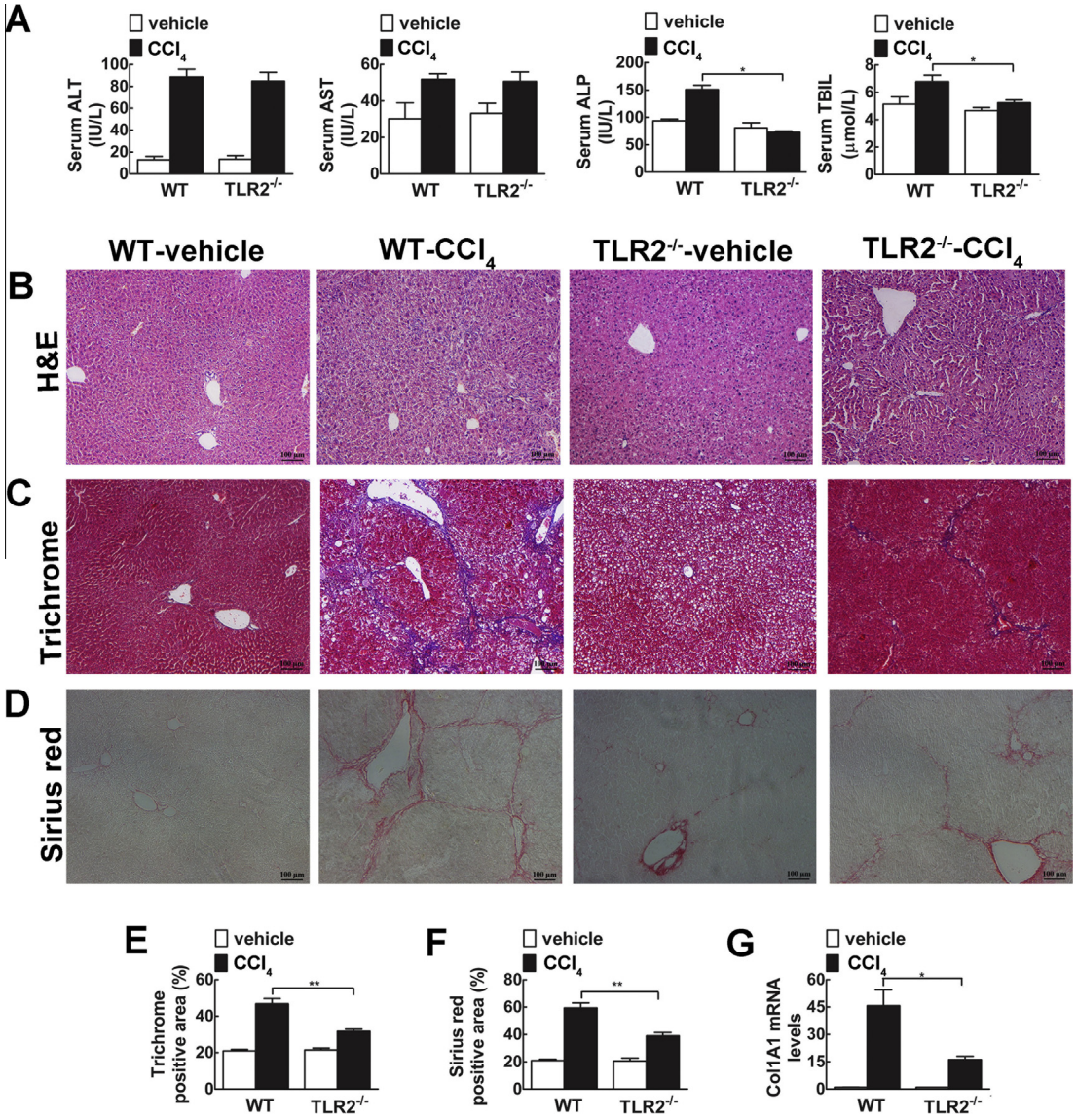

Fig. 1. CCl_4-induced liver injury and collagen accumulation are diminished in $TLR2^{-/-}$ mice. (A) Liver injury was measured by Serum ALT, AST, ALP and TBIL. (B) Hematoxylin and eosin (H&E) staining of liver sections from WT and $TLR2^{-/-}$ mice treated with vehicle or CCl_4 (original magnification, ×100). (C and D) Representative images of Masson's trichrome and Sirius red staining of livers from WT and $TLR2^{-/-}$ mice treated with vehicle or CCl_4 (original magnification, ×100). (E and F) Histomorphometric analysis of stained sections, expressed as percentage of positive pixels. (G) Liver Col1A1 messenger RNA levels were determined by quantitative polymerase chain reaction. Values represent means ± SEM for 9 vehicle and 10 CCl_4 treated mice. $^*P < 0.05$, $^{**}P < 0.001$.

treatment were significantly attenuated in $TLR2^{-/-}$ mice (Fig. 1B). Masson's trichrome and Sirius red staining for fibrosis (Fig. 1C–F) also demonstrated a marked decrease in CCl_4-induced ECM collagen deposition in $TLR2^{-/-}$ mice compared with WT mice. This was further confirmed by the expression of type I procollagen α1 chain (Col1A1) mRNA (Fig. 1G), which encodes the pro-alpha1 chains of type I collagen. Taken together, these results suggested that TLR2 could mediate CCl_4-induced liver injury and collagen accumulation.

3.2. TLR2 deficiency inhibits hepatic stellate cell activation upon CCl_4 administration

In view of the fact that activated HSC was considered as the main source of collagen in liver tissue, we then performed liver immunohistochemistry for the activated stellate cell marker α-SMA (Fig. 2A). Compared with WT littermates administrated of CCl_4, less α-SMA-positive cells were observed in the periductular regions of $TLR2^{-/-}$ fibrotic animals. This is in agreement with those obtained by semiquantitative assays of α-SMA mRNA levels (Fig. 2B) and Western blot analysis of α-SMA protein expression (Fig. 2C). Simultaneously, HSCs isolated from $TLR2^{-/-}$ mice also exhibited reduced pro-inflammatory response after TGF-β1 stimulation in vitro (Supplementary Fig. 1A–D). Collectively, these findings indicated that TLR2 could decrease liver collagen deposition through inhibiting the activation of HSCs.

3.3. TLR2 deficiency decreases inflammatory infiltration and reduces mRNA level of profibrotic and proinflammatory cytokines in liver upon CCl_4 administration

Inflammatory mediators from Kupffer cells have been proved to play a pivotal role in HSCs activation and fibrogenesis [21]. To explore whether TLR2 regulate inflammatory infiltration during fibrosis, we performed F4/80 immunohistochemistry staining to determine macrophages infiltration. Our result showed that TLR2

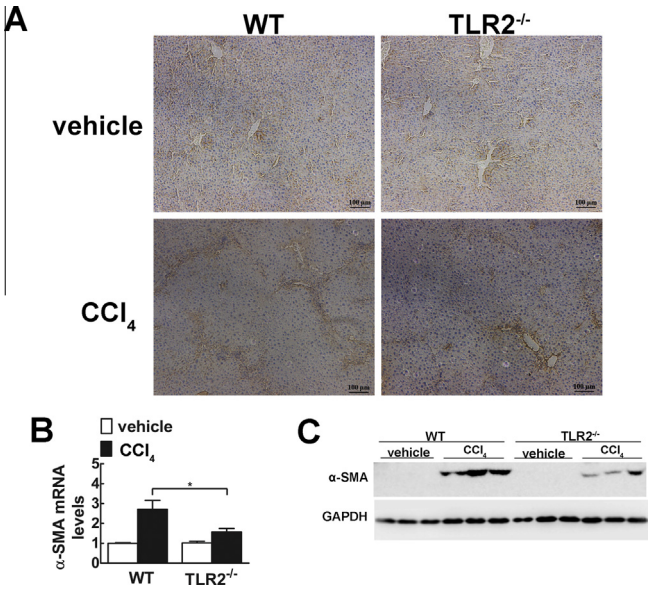

Fig. 2. CCl_4-induced hepatic stellate cell activation is ameliorated in $TLR2^{-/-}$ mice. (A) Immunohistochemical staining of α-SMA in the livers of WT and $TLR2^{-/-}$ mice treated with vehicle or CCl_4 (original magnification, ×100). (B) Real-time quantitative PCR analysis of α-SMA mRNA expression in groups. (C) Expression of α-SMA was determined by Western blot analysis. Values represent means ± SEM for 9 vehicle and 10 CCl_4 treated mice. $^*P < 0.05$.

deficiency reduced CCl_4-induced macrophages infiltration (Fig. 3A). Immunohistochemistry staining of neutrophils (Gr-1), CD4 T lymphocytes and CD19 B lymphocytes also demonstrated the anticipated alterations (Supplementary Fig. 2).

In response to liver damage caused by CCl_4, proinflammatory factors such as TNF-α, IL-6, MCP-1 Ccl4, CXCL-1 and CXCL-2 were upregulated in liver. Quantitative R'T-PCR analysis and ELISA analysis revealed a strong suppression of pro-inflammatory response in $TLR2^{-/-}$ mice as compared with WT mice after treatment of CCl_4 (Fig. 3B and Supplementary Fig. 3). Similarly, the hepatic expression of profibrotic molecules, including TGF-β, TIMP1 and PDGFR, were also down-regulated in CCl_4-treated $TLR2^{-/-}$ mice (Fig. 3C). Meanwhile, we further tested the expression of TLR4 and found that the mRNA and proteins levels of TLR4 were decreased in $TLR2^{-/-}$ mice compared to WT mice after CCl_4 treatment (Supplementary Fig. 4).

3.4. TLR2 deficiency attenuates mitogen-activated protein kinases (MAPK) phosphorylation and NF-κB activation in liver upon CCl_4 administration

To identify potential molecular mechanisms of TLR2-mediated pro-fibrogenic effect during CCl_4-induced liver fibrosis, several well-documented signaling pathways connected with liver fibrosis were investigated in liver [22,23]. Though equal amounts of ERK1/2 protein were detected, CCl_4-induced ERK1/2 phosphorylation was significantly diminished in $TLR2^{-/-}$ mice compared with WT mice (Fig. 4A). Likewise, CCl_4-induced phosphorylation of p38 and JNK were also significantly decreased in $TLR2^{-/-}$ mice compared with WT mice, affirming attenuated proinflammatory signaling (Fig. 4B and C). Moreover, we observed impaired NF-κB activation in CCl_4-treated $TLR2^{-/-}$ mice compared with CCl_4-treated WT mice. The similar pattern was observed in in vitro activated HSCs and macrophages isolated from wild-type and $TLR2^{-/-}$ mice (Supplementary Fig. 5). Altogether, these results

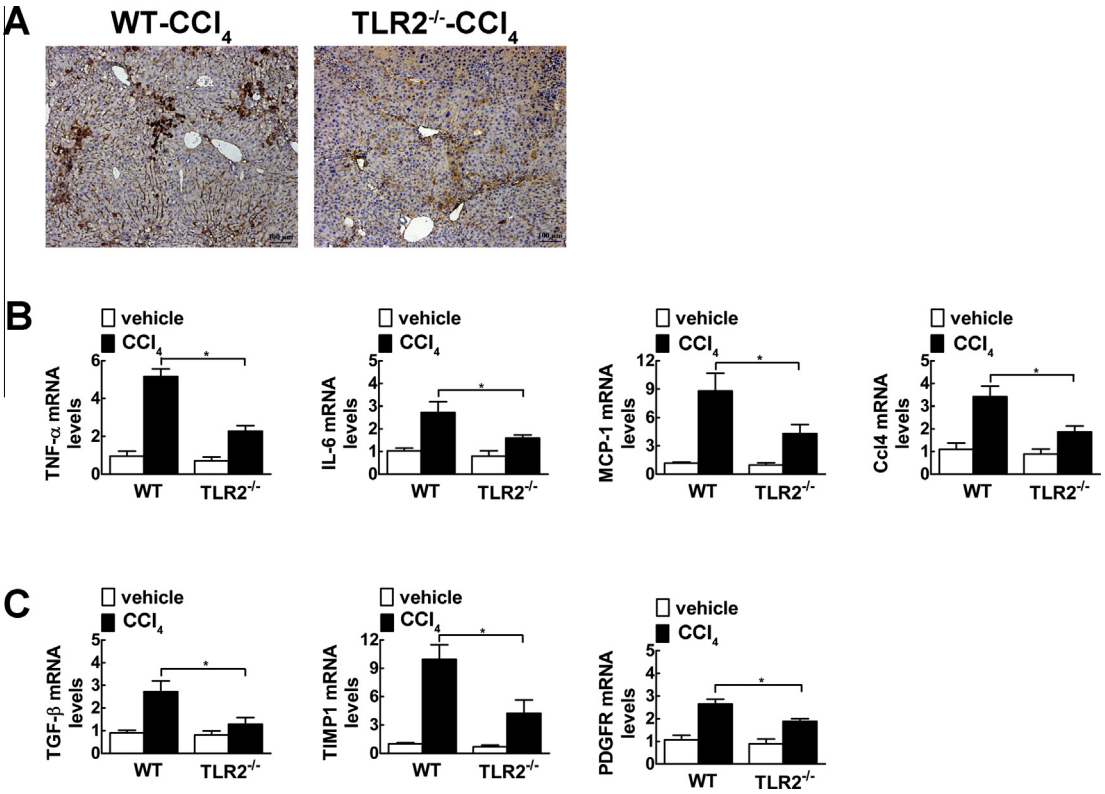

Fig. 3. TLR2 deficiency protects against CCl_4-induced hepatic inflammation and fibrogenesis. (A) Macrophages infiltration after injection of CCl_4 were evaluated by F4/80 immunohistochemistry (original magnification, ×100). (B) Real-time quantitative PCR analysis of hepatic expression of pro-inflammatory genes including TNF-α, IL-6, MCP-1 and Ccl4. (C) Real-time quantitative PCR analysis of hepatic expression of profibrotic genes including TGF-β, TIMP1 and PDGFR. Values represent means ± SEM for 9 vehicle and 10 CCl_4 treated mice. $^*P < 0.05$.

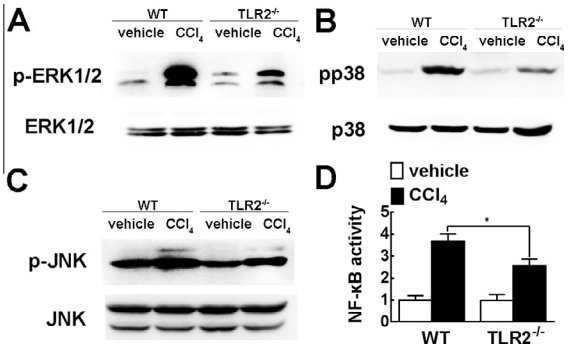

Fig. 4. CCl_4-induced MAPK and NF-κB activation is attenuated in $TLR2^{-/-}$ mice. Phosphorylation of (A) ERK1/2, (B) P38 and (C) JNK was determined by Western blot analysis in lysed liver tissue. (D) NF-κB activation was assessed in nuclear extracts. Values represent means ± SEM for 9 vehicle and 10 CCl_4 treated mice. $^*P < 0.05$.

suggest that TLR2 may promote the fibrogenesis via activating MAPK and NF-κB signaling pathways.

4. Discussion

Liver fibrosis occurs in virtually many types of chronic liver diseases and is characterized by a reversible accumulation of collagenous matrix and sustained inflammation [10,24,25]. In view of its crucial role in regulation of inflammation even under injury and wound healing, TLR2 has been implicated in a number of chronic liver diseases [10]. To date, a series of studies about the relationship between TLR2 and liver fibrosis have been published, but no clear consensus has been reached [7,17]. Especially, the in-depth molecular link between TLR2 signaling and chronic liver fibrosis remains elusive. In previous studies, Hartmann et al. [26] employed bile duct ligation (BDL) mice to demonstrated that TLR2 signaling on monocytes in the lamina propria is important in intestinal inflammation and bacterial translocation that contributed to liver fibrosis. In addition, Moles et al. [27], using an acute liver injury mouse model, showed that TLR2 is required for optimal recruitment of neutrophils to the hepatic parenchyma, but is dispensable for subsequent wound-repair/fibrogenesis and regenerative responses. However, Seki et al. [7] observed that TLR4, but not TLR2, are required for BDL-induced hepatic fibrosis. Hence, in the current study, we analyzed liver fibrosis marker and fibrosis-related molecular alterations in a model of CCl_4-induced fibrosis. Using this model, we found that TLR2 deficiency protected against the HSCs activation and liver fibrosis mainly through inhibiting MAPK and NF-κB signaling pathways.

Administration of CCl_4 in mice duplicates the liver damage, activation of HSCs and liver fibrosis observed in chronic human liver disease. Our data indicated that accumulation of collagen in CCl_4-treated $TLR2^{-/-}$ mice was significantly reduced as compared with that in WT mice (Fig. 1C and D), as well as the α-SMA production (Fig. 2). Interestingly, following injection of CCl_4, liver injury as measured by ALT and AST levels was not significantly different in wild-type and $TLR2^{-/-}$ mice (Fig. 1A), indicating that TLR2 does not mediate CCl_4 hepatotoxicity but fibrosis progression. Meanwhile, ALP and TBIL levels were reduced in $TLR2^{-/-}$ mice relative to WT mice following CCl_4 treatment (Fig. 1A), suggesting that TLR2 may contribute to CCl_4-induced bile duct injury. Though the expression level of TLR2 in hepatocytes is very low and its responses are fairly weak in vivo, non-parenchymal liver cells, such as the Kupffer cells, HSCs and biliary epithelial cells, express relatively abundant level of TLR2 [28]. It is evident that non-parenchymal liver cells are primarily responsible for the production of ECM components. And they are also considered to be the main source of profibrotic molecules and proinflammatory factors [15]. In agreement with previous studies that TLR2 activation induced profibrotic molecules and proinflammatory cytokines production in non-parenchymal liver cells in vitro [29,30], We found that the protection effect of TLR2 deficiency on CCl_4-induced liver fibrosis was accompanied by downregulating of proinflammatory and profibrotic mediators including TNF-α, IL-6, MCP-1, Ccl4, CXCL-1, CXCL-2, PDGFR, TGF-β and TIMP1 in vivo (Fig. 3B and C and Supplementary Fig. 2). Moreover, TLR2 deficiency was associated with less hepatic inflammation, as determined by reduced macrophages and neutrophils infiltration in CCl_4-induced liver fibrosis (Fig 3A and Supplementary Fig. 2).

The MAPK family includes extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Once these molecules are activated, they recruit to Ras, which leads to the transcription of cell-proliferative and profibrogenic factors. MAPKs have been reported to be critical for HSCs activation and collagen synthesis. Suppression of ERK activation was associated with complete inhibition of HSCs proliferation in vitro [31]. JNK inhibition prevented TGF-β induced murine HSCs activation and decreased TGF-β signaling in human HSCs in vitro [32]. JNK inhibition also significantly reduced CCl_4-induced liver fibrosis in vivo [32]. In addition to MAPKs, NF-κB, a master regulator of inflammation and cell death, also plays a key role in regulating the survival of hepatocytes, inflammation in Kupffer cells, and survival, inflammation and activation in HSCs [33]. The crucial role of NF-κB in the liver is underlined by the fact that NF-κB inhibitors block hepatic fibrogenesis in experimental models of liver fibrosis [34]. In the present study, we found that TLR2 deficiency attenuated activities of MAPK and NF-κB in in vitro activated HSCs and macrophages (Supplementary Fig. 5). MAPK and NF-κB signaling were activated after CCl_4 treatment in vivo, which is in line with previous reports. Interestingly, TLR2 deficiency significantly inhibited CCl_4-induced activation of MAPK and NF-κB (Fig. 4), suggesting that TLR2 is critical for the activation of MAPK and NF-κB in the mouse model of CCl_4-induced hepatotoxicity.

In summary, our studies show that HSCs activation and inflammation response during CCl_4-induced liver fibrosis are associated with TLR2 mediated MAPK and NF-κB signaling pathways. These results introduce a new aspect of TLR2 biology potentially related to the pathogenesis of liver fibrosis. Thus, TLR2 inhibition appears to be a promising strategy for the prevention of hepatic fibrosis in patients with chronic liver disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.04.042.

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